A Drosophila Axin homolog, Daxin, inhibits Wnt signaling

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Summary: Daxin, Axin, Wnt signaling, Drosophila melanogaster

INTRODUCTION

Like in many other signaling pathways, most components in Wnt signal transduction are highly conserved in evolution. In organisms ranging from C. elegans to mice, a similar hierarchy of signaling molecules transmits the Wnt signal to the nucleus. In current models of this pathway, the secreted Wnt protein is received by Frizzled-like cell surface receptors. In the cytoplasm, Dishevelled (Dsh) relays the signal to a complex of several proteins, including the protein kinase glycogen synthase kinase-3β (GSK-3β) and β-catenin (see URL: http://www.stanford.edu/~rnusse/wntwindow.html and reviewed by Cadigan and Nusse, 1997). In this complex, the β-catenin protein becomes targeted for degradation after being phosphorylated by GSK-3β (Aberle et al., 1997; Yost et al., 1996). After Wnt signaling and the resulting down-regulation of GSK-3β kinase activity (Cook et al., 1996), β-catenin escapes from degradation. β-catenin then can form a complex with the nuclear DNA binding protein TCF (Behrens et al., 1996; Molenaar et al., 1996; Morin et al., 1997) and participate in transcriptional activation of Wnt target genes (Korinek et al., 1998).

Most components of the Wnt signaling pathway were identified in genetic screens in Drosophila, where the Wnt gene wingless (wg) patterns the embryo through the β-catenin homolog armadillo (arm). However, other molecules that regulate β-catenin levels have emerged from mammalian systems. APC, for example, which binds β-catenin to facilitate its degradation, was discovered as a human tumor suppressor gene (reviewed by Polakis, 1997). Subsequent cloning of the Drosophila homolog of APC showed that it is involved in regulating Arm in photoreceptor cells in the eye (Ahmed et al., 1998). Wnt signaling in the C. elegans embryo is also regulated by an APC related protein, Apr-1 (Rocheleau et al., 1997).

Another component in β-catenin regulation is the vertebrate Axin protein, which binds to β-catenin, GSK-3β and APC (Behrens et al., 1998; Hart et al., 1998; Ikeda et al., 1998; Itoh et al., 1998; Kishida et al., 1998; Nakamura et al., 1998; Sakana et al., 1998; Yamamoto et al., 1998). Axin is the product of a mouse gene, fused, that affects embryogenesis (Zeng et al., 1997). An Axin-related vertebrate protein, Conductin/Axil, has been identified as a binding partner for β-catenin (Behrens et al., 1998; Yamamoto et al., 1998). Axin and Conductin overexpression phenotypes in Xenopus indicate that these proteins are negative regulators of Wnt signaling (Behrens et al., 1998; Itoh et al., 1998; Zeng et al., 1997), in agreement with the biochemical interactions between Axin and β-catenin. The consequences of loss of function of Axin are not entirely clear; there are several alleles of the mouse fused gene but it is not established whether these are nulls (Vasicek et al., 1997). Other Axin-like genes in Drosophila or C. elegans would provide important information on their genetic requirements in Wnt signaling, but it is only recently that they have been identified. The worm genome actually may not contain an Axin homolog (Ruvkun and Hobert, 1998).

We therefore were interested in identifying Axin in the fly. By screening the Drosophila EST database, we have found a fly Axin homolog, Daxin. Using double-stranded RNA interference methods, we generate a loss-of-function phenotype and show that loss of Daxin in the embryo mimics overexpression of wg and of arm. Conversely, overexpression of Daxin is similar to loss of wg. In addition to interfering with the Wg signal, we show that Daxin overexpression can block the DWnt-2 signal. We have also examined the Daxin protein and show that in embryo lysates it interacts with Arm and...
Zeste-white 3 (Zw3 is the fly homolog of GSK-3β). After submission of the first version of this manuscript, a paper describing a loss-of-function allele in a fly homolog of Axin was published (Hamada et al., 1999); our studies complement and extend their findings.

MATERIALS AND METHODS

Cloning of Daxin
A Drosophila expressed sequence tag (EST) (clone AA699261) with strong homology to the DXD domain of mouse Axin was identified in the database of GenBank EST division by a BLASTN search with mouse Axin. Two primers were designed to this EST: one to the 5′ end of the EST (CCGAATTCAGCAGCTGCTCTTGAGCA-GCA) and one at the site of the stop codon (CCGCTGCAGATGCGTGTACAGACGACCATCG). These primers were used to amplify a 330 bp fragment from reverse transcribed total embryonic RNA. This fragment was ligated into the pBluescript(SK−) vector and from the resulting plasmid (pBluescript(SK−)Daxin) oligos were designed that spanned the RGS domain of Daxin (CGAAGAATTCCTGCGCACTTACTCAGGATGCTGCAGCC-GAG) and used these oligos in a QuiChanger site-directed mutagenesis (Stratagene) reaction on myc-tagged Daxin (form A1) in pBluescript. Wild-type Daxin (forms A1 and A2), myc-tagged Daxin and DaxinRGS were cloned into pUAST vector and used for generation of UAS-transgenic flies. Wild-type Daxin (form A2) was also cloned into the pMK33HS plasmid and used for generation of UAS-transgenic flies. Wild-type Daxin (form A1) and a 2235 bp open reading frame were generated to replace the wild-type carboxy terminus of the Daxin protein, encoding the myc-tagged carboxy terminus was subsequently cloned to generate pBluescript(SK−)DaxinRGS. The 330 bp fragment above was also cloned into a vector that already contained a 4166 bp pUAST construct (Brand and Perrimon, 1993) containing wild-type Daxin (form A2-12) and a 2217 bp open reading frame (form A1) and a 2335 bp open reading frame (form A2).

Daxin constructs
The 330 bp fragment above was also cloned into a vector that already contained a myc tag sequence so that the open reading frame of this fragment carried a myc tag at the carboxy terminus. This fragment encoding the myc-tagged carboxy terminus was subsequently cloned to replace the wild-type carboxy terminus of the Daxin gene (form A1). In order to construct the RGS deletion in Daxin we designed two complementary oligos that spanned the RGS domain of Daxin (CGAAGAATTCCTGCGCACTTACTCAGGATGCTGCAGCC-GAG) and used these oligos in a QuiChanger site-directed mutagenesis (Stratagene) reaction on myc-tagged Daxin (form A1) in pBluescript. Wild-type Daxin (forms A1 and A2), myc-tagged Daxin and DaxinRGS were cloned into pUAST vector and used for generation of UAS-transgenic flies. Wild-type Daxin (form A2) was also cloned into the pMK33HS vector (Koelle et al., 1991) and further modified by Chi-hwa Wu, R. N. to include the heat-shock promoter) for overexpression in Schneider 2 cells.

RNA interference and cuticle preparations
Sense and antisense Daxin RNA was generated from linearized pBluescript(SK−) Daxin (A1). The double-stranded RNA was annealed as described by Kennerdell and Cartewh (1998) and injected posteriorly into w+1118 host embryos at a concentration of 2 μM. After 48 hours at 18°C, the embryos were carefully transferred with a wire loop to an Eppendorf tube. The embryos were fixed in a total volume of 1.4 ml containing 700 μl heptane, 560 μl phosphate-buffered saline, and 140 μl 37% formaldehyde for 2 minutes. To devitellinize the embryos, the lower aqueous phase was removed and replaced with 700 μl methanol and shaken for 2 minutes, then washed 3 times with methanol. The embryos were mounted in Hoyer’s lactate acid (1:1) and incubated at 60°C for 12 hours and then visualized by dark-field microscopy.

Fly stocks, crosses, and generation of transgenic UAS-Daxin flies
pUAST constructs (Brand and Perrimon, 1993) containing wild-type Daxin (forms A1 and A2), myc-tagged Daxin and DaxinRGS were introduced into w+1118 hosts by P-element-mediated transformation using standard methods (Rubin and Spradling, 1982). UAS-wg; UAS-Daxin (A2-12/TM3) and UAS-DaxinRGS (D3-3/TM3) were crossed to the embryonic driver Da-Gal4 (Georgias et al., 1997), (Fig. 3A-C); UAS-Daxin (A2-63/CyO) and UAS-DaxinRGS (D3-3) were crossed to 69B-Gal4 (Brand and Perrimon, 1993), (Fig. 3E-F); UAS-Daxin (A2-28/CyO) was crossed to GMR-Gal4 (Freeman, 1996), (Fig. 4C) and to GMR-Gal4, UAS-wg/CyO (Fig. 4D). The wg mutant embryo in Fig. 2B carries the w+1118 allele which is a small deficiency and is RNA and protein null (van den Heuvel et al., 1993).

The effects of Daxin and ΔTCF on Dwnt-2 signaling were performed by making ‘flip-out’ clones (Pignoni and Zipursky, 1997) in ovaries to examine the emergence of pigment cells (Kozopas et al., 1998). Flies carrying the following transgenes were used: (i) UAS-Dwnt-2, (ii) UAS-Dwnt-2; ΔTCF, (iii) UAS-Dwnt-2; UAS-Daxin (A2-4), (iv) UAS-Dwnt-2; UAS-Daxin (A2-12). These stocks were crossed to flies carrying wpw act>cd2>G4w+1; UAS-GFP; hsFLP: MKRIS/TM6. Larvae at the mid-third instar stage were heat shocked at 35°C for 1 hour to induce expression of FLP. Larvae were grown at 29°C until eclosion. Two days after eclosion, ovaries were removed from females and mounted live under coverslips for examination of pigment cells as described previously (Kozopas et al., 1998).

Embryos for protein analysis (Fig. 5) were of Canton-S stock.

Generation of anti-Daxin antibodies
The 330 bp PCR fragment above was also cloned into the pcR2.1 vector using the TA cloning kit (Invitrogen). Sequencing of this insert revealed that the EST sequence obtained from GenBank lacked a couple of bases near the 5′ end. Sequencing of several individual clones isolated from the library above revealed that the sequence deposited into GenBank was indeed incorrect. The 330 bp EcoRI fragment in pcR2.1 was subsequently cloned into the GST-fusion protein plasmid pGEX4T-2 (Pharmacia). GST fusion protein was produced and purified as described by the vendor and used as immunogen in rabbits. Antiserum were affinity purified against the immunogen which was covalently immobilized to Sepharose. A second antiserum was obtained by immunizing rats with a GST fusion to a 315 amino acid portion of the Daxin protein spanning the RGS domain (a 945 bp EcoRI fragment from Daxin was ligated into the EcoRI site of plGEX4T-3). Both antiserata reacted with the same set of bands with apparent molecular weight of 95 kDa on an immunoblot.

Generation of anti-Zw3 antibodies
An EcoRV-Smal fragment of zw3 cDNA (pBOV1 plasmid kindly provided by Esther Siegfried) was cloned into the blunt EcoRI site of plGEX-2T (Pharmacia). GST-Zw3 fusion protein was produced and purified as described by the vendor and used as immunogen in rabbits. Antiseras were affinity purified against the immunogen which was covalently immobilized to Sepharose. A second antiserum was obtained by immunizing rats with a GST fusion to a 315 amino acid portion of the Daxin protein spanning the RGS domain (a 945 bp EcoRI fragment from Daxin was ligated into the EcoRI site of plGEX4T-3). Both antiserata reacted with the same set of bands with apparent molecular weight of 95 kDa on an immunoblot.

Cell culture, lysate preparation, immunoblotting and immunoprecipitations
Schneider 2 (S2) cells were cultured in Schneider's Drosophila medium (Gibco BRL) supplemented with 12.5% fetal bovine serum (Sigma) and penicillin and streptomycin. pMK33HS-Daxin was introduced into S2 cells using calcium phosphate mediated transfection followed by selection with 150 μg/ml hygromycin B (Gibco BRL) until a hygromycin-resistant cell line was established. The Daxin transgene was induced by a 30 minute heat shock at 37°C and then cultured for an additional 3 hours before lysate preparation. For preparation of lysates (Fig. 5C), cells were first washed in phosphate-buffered saline and then lysed on ice with lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1% Triton X-100, pH 8) supplemented with protease inhibitors (1 mM Pefabloc, 0.5 μg/ml leupeptin, 1 μg/ml pepstatin A) and phosphatase inhibitors (0.4 μM microcystin-LR, 1 μM vanadate). Nuclei and insoluble proteins were pelleted at 20,000 g at 4°C for 10 minutes. Protein concentrations were determined using the Bio-Rad Protein Assay dye reagent. For preparation of lysates
from embryos, 0- to 8-hour old embryos (Fig. 5A and B) or embryos of the indicated age (Fig. 5D) were collected, dechorionated in 50% bleach for 2 minutes and lysed in ice cold lysis buffer supplemented with protease inhibitors and phosphatase fitting dounce. Insoluble proteins were pelleted and protein concentrations were determined as above. Per lane on SDS-PAGE, 20 μg of total protein was resolved after boiling 5 minutes in SDS-PAGE sample buffer (50 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol, bromophenol blue). For immunoprecipitations, 200 μg total protein was incubated overnight at 4°C with 1 μl of either preimmune serum or affinity purified rabbit anti-Daxin antibody, 10 μl of either pre-immune or rat anti-Axin antibody, 10 μl affinity purified rabbit anti-Zw3 antibody, or 20 μl mouse monoclonal anti-Arm (7A1; Peifer, 1993) antibody and 10 μl of a 1:1 slurry of either protein A Sepharose or protein G Sepharose. Immune-complexes were washed four times with lysis buffer, boiled in SDS-PAGE sample buffer, and resolved by SDS-PAGE. Proteins were then transferred to nitrocellulose, and blots were blocked in blocking buffer (3% nonfat dry milk, 1% BSA in TBST [20 mM Tris-HCl, 150 mM NaCl, 0.2% Tween-20, pH 8]), and then incubated overnight at 4°C in blocking buffer with rat or rabbit anti-Axin antibody, mouse

Fig. 1. Diagrammatic presentation and sequence alignment of Daxin. (A) Diagram of Daxin structure. The domain structure of Daxin. (Form A1) is presented as a stick diagram. Form A2 contains an additional six amino acids at the indicated position. The most highly conserved RGS and DIX domains are labelled and presented as shaded boxes. Homologies of these two domains to corresponding domains of Axin and Conductin are indicated, suggesting that Daxin is more closely related to Axin than to Conductin. The Arm binding motif was mapped previously (Hamada et al., 1999), while the exact boundaries of the Zw3 binding domain have not been determined. The asterisk indicates the position of two additional serine residues not present in the sequence determined by Hamada et al. (1999). (B) Protein sequence alignment of Daxin, mouse Axin and Conductin. Amino acids in Daxin identical and conserved to those of Axin and Conductin are highlighted in gray. The RGS and DIX domains are marked by a black and a blue box, respectively. The GSK-3β/putative Zw3 and β-catenin/Arm binding sites of Daxin, Axin and Conductin are boxed in red and green, respectively. The sequences, boxed with a dashed line, in Daxin indicate amino acid differences between two independent Daxin isolates and the previously published Daxin sequence (Hamada et al., 1999). Form A2 includes the six amino acids SRSQSS while Form A1 does not. The amino acids, boxed with a dashed line, in Axin indicates the sequence not present in Form 1 (Zeng et al., 1997).
We identified a homolog of Drosophila Axin gene by searching the EST database with the protein sequence of mouse Axin. An EST with significant homology to the DIX (similar between Axin and Dishevelled) domain of Axin was identified and used to isolate the full-length clone from an embryonic Drosophila cDNA library.

The complete sequence of Daxin cDNA is 4669 bp in length and contains two alternative polyadenylation sites and an open reading frame encoding a 739 amino acid protein (form A1; Fig. 1A and B). Several clones (3 out of 5) contained a 18 basepair (bp) insert (form A2). This insert is in a similar, albeit not identical, region where mouse Axin contains a 108 bp insert to give rise to two different forms (form 1 and 2). The 18 nucleotides in the fly gene code for six amino acids, four of which are serine residues, directly amino-terminal to the DIX domain. No difference in the activities of the two isoforms of either mouse Axin or Daxin have been identified. The recently published sequence of Daxin (Hamada et al., 1999) is identical to form A2, but lacks two serine residues at position 644 and 645. The differences in sequences, including forms A1 and A2, may reflect naturally occurring polymorphisms in the Daxin gene.

Overall, the identity between mouse and Drosophila Axin is 21% although several domains are more highly conserved (Fig. 1A and B). The Daxin protein is more distantly related (17%) to Conductin (Behrens et al., 1998), suggesting that it is the vertebrate Axin ortholog. Like the vertebrate Axin proteins, Daxin contains an RGS domain near the amino terminus. These domains are found in a family of proteins that regulate G-proteins. Although no such G-protein signaling function has been attributed to the mouse Axin RGS domain (Mao et al., 1998), the RGS has been shown to be required for the interaction with the APC protein (Behrens et al., 1998). Deletion of this domain in mouse Axin produces a dominant negative Axin protein, as assayed by axis duplication activity in Xenopus embryos (Itoh et al., 1998; Zeng et al., 1997). In contrast, overexpression of wild-type mouse Axin inhibits axis duplication, suggesting that interaction with APC is crucial for Axin’s ability to block the Wnt signaling pathway.

The region between the RGS and DIX domains, where both GSK-3β and β-catenin binding sites are located in Axin and Conductin, contains stretches of lower similarity. Despite this low similarity, Daxin does in fact interact with both Arm and Zw3 (see below). Hamada et al. mapped the Arm binding domain in Daxin to amino acids 459-538 by precipitating in vitro translated Arm with GST-Daxin fusion proteins (Hamada et al., 1999), a region which contains significant homology with Axin and Conductin. The Daxin protein contains four potential nuclear localization signals, but the mouse Axin protein, which also contains a nuclear localization signal, has been localized predominantly to the cytoplasm (Behrens et al., 1998; Fagotto et al., 1999). The Daxin gene is located on the third chromosome at 99D as determined by hybridization to polytene chromosomes (M.F., unpublished data; Hamada et al., 1999).

### RESULTS

**Isolation of a Drosophila Axin gene**

We identified a Drosophila homolog of Axin (Daxin) by anti-Zw3 (Ruel et al., 1993), or mouse anti-Arm at dilutions of 1:1000, 1:2000, or 1:500, respectively. Proteins were detected using HRP-conjugated secondary antibodies (Bio-Rad) with the ECL western blot detection reagents (Amersham).

**Disruption of Daxin function by RNA interference**

It has been shown previously that loss-of-function mutant phenotypes can be mimicked by the injection of double stranded RNA. This method, referred to as RNA interference (RNAi), was initially successfully used in C. elegans and more recently in Drosophila (Kennerdell and Carthew, 1998). The mechanism by which RNAi specifically interferes with gene function is not well understood but it has been speculated that it acts by directing the endogenous mRNA for degradation (reviewed by Sharp, 1999; Tabara et al., 1998).

We used RNAi to disrupt the function of Daxin during patterning of the embryonic cuticle, a process which requires Wg signaling. The wild-type embryo secretes a cuticle consisting of a repeated pattern of denticle belts with intervening naked regions (Fig. 2A). Loss of Wg leads to a cuticle covered with denticles and lacking naked areas (Fig. 2B and Nüsslein-Völlhard and Wieschaus, 1980). Overexpression of Wg in the embryo leads to loss of all denticle structures, i.e. a naked cuticle (Fig. 3A and Noordermeer et al., 1992). We found that disruption of Daxin expression by RNAi leads to a similar naked cuticle (Fig. 2C and D), suggesting that Daxin functions to down-regulate Wg signaling. Virtually all injected embryos had extra naked cuticle, with a range from partially (Fig. 2D) to nearly completely naked (Fig. 2C). Control injection of either sense or antisense single stranded Daxin RNA did not cause any phenotypic changes (data not shown). As expected, wg RNAi produced a partial wg-like cuticle (Kennerdell and Carthew, 1998; and data not shown). Thus, mimicking loss of Daxin function by RNAi leads to phenotypes similar to overexpressing wg, consistent with the model that Daxin is a negative regulator of Wg signaling.

In a paper by Hamada et al., a P-element insertion near the beginning of the Daxin gene disrupts expression of the gene to produce a loss-of-function allele of Daxin (Hamada et al., 1999). Embryos lacking zygotic Daxin produce a loss-of-function allele of Daxin (Hamada et al., 1999). Embryos lacking zygotic Daxin are still wild type and only upon removal of the maternally contributed Daxin gene product is a naked cuticle revealed. Thus, our RNAi experiments successfully disrupt the maternally contributed Daxin gene product and produce a phenotype identical to that of a loss-of-function mutation in the gene.

**Overexpression of Daxin blocks Wg signaling in the embryo and wing**

To address further whether Daxin regulates Wg signaling, we used the UAS-Gal4 system to overexpress Daxin in various tissues. The Daughterless (Da)-Gal4 driver expresses early during embryogenesis (Georgias et al., 1997) and when combined with UAS-wg produces a completely naked cuticle (Fig. 3A and Noordermeer et al., 1992). Overexpression of Daxin using the Da-Gal4 driver produced a loss of wg-like phenotype, a cuticle covered with denticles (Fig. 3B, compare with Fig. 2B), consistent with overexpression of Daxin blocking Wg signaling in the embryonic epidermis.

To extend this study we misexpressed Daxin in the wing using the 69B-Gal4 driver. wg is expressed in a narrow stripe along the presumptive wing margin where it is required for proneural achaete-scute complex gene expression and for the
formation of margin bristles (Couso et al., 1994; Phillips and Whittle, 1993; Rulifson et al., 1996). Loss of Wg signaling along the wing margin, as in the case of dsh loss-of-function clones, leads to loss of these margin bristles and notches along the wing (Rulifson et al., 1996). Overexpression of Daxin in the wing also produces this wing notching effect (Fig. 3E), a result consistent with Daxin interfering with the Wg signaling pathway at the wing margin.

Deletion of the RGS domain of mouse Axin produces a dominant negative Axin protein, as assayed by its ability to induce a secondary axis when overexpressed in Xenopus embryos (Itoh et al., 1998; Zeng et al., 1997). We constructed an analogous mutation in Daxin which deletes the entire RGS domain (DaxinΔRGS; deletion of amino acids 50 to 172). Surprisingly, when overexpressed, this Daxin mutant produces the same phenotypes in the embryo (Fig. 3C) and in the wing (Fig. 3F) as does wild-type Daxin overexpression. Thus, the RGS domain is dispensable in overexpression assays for Daxin.

Overexpression of Daxin modifies ectopic Wnt effects in the eye and the ovary

The above data clearly demonstrate that Daxin loss-of-function or overexpression produces phenotypes similar to wg overexpression or loss-of-function, respectively. However, it fails to demonstrate that Daxin can modify effects of Wnt signaling. To examine whether ectopic Daxin expression can interfere with the effects of Wnt signaling, we misexpressed wg in the eye and DWnt-2 in the ovary.

When overexpressed in the eye, wg produces a glassy eye (Fig. 4B) that is greatly reduced in size compared to a wild-type eye (Fig. 4A). Misexpression of Daxin in the eye produces a weakly rough eye (Fig. 4C). When Daxin and wg are both overexpressed, the morphology of the eye is intermediate to that of a wild-type eye and that of a wg eye, indicating that Daxin can interfere with the Wg signal.

In order to address whether Daxin inhibits Wg only or whether it can function as a more general inhibitor of Wnt signals, we examined whether Daxin could interfere with a different Wnt signal. Four Wnt genes have been identified in Drosophila (see URL: http://www.stanford.edu/~rnusse/wntwindow.html), but...
mutations have been described only for two of them, wg and DWnt-2 (Kozopas et al., 1998; Nüsslein-Völlhard and Wieschaus, 1980). Loss of DWnt-2 produces a muscle migration defect in the male gonads, resulting in male sterility, and a lack of the characteristic pigment cells that migrate over the male testis (Kozopas et al., 1998). Ovaries are normally not surrounded by pigment cells, but misexpression of the male testis (Kozopas et al., 1998). Ovaries are normally not surrounded by pigment cells, but misexpression of the male testis (Kozopas et al., 1998). Ovaries are normally not surrounded by pigment cells, but misexpression of the male testis (Kozopas et al., 1998).

Table 1. Daxin misexpression interferes with DWnt-2-induced pigment cell formation on the ovary

<table>
<thead>
<tr>
<th>Transgene(s)</th>
<th>% pigment cell formation (n)</th>
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<tr>
<td>UAS-DWnt-2</td>
<td>91 (99)</td>
</tr>
<tr>
<td>UAS-DWnt-2; UAS-ΔNdTCF</td>
<td>21 (102)</td>
</tr>
<tr>
<td>UAS-DWnt-2; UAS-Daxin (A2-4)</td>
<td>20.8 (96)</td>
</tr>
<tr>
<td>UAS-DWnt-2; UAS-Daxin (A2-12)</td>
<td>20 (117)</td>
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Clonal expression of DWnt-2 alone or in combination with either ΔNdTCF or Daxin was initiated by heat-shocking mid-third instar stage embryos at 35°C for one hour to induce expression of FLP. GFP expressing ovaries from adults were dissected and scored for the presence or absence of pigment cells. n=total number of adults containing GFP positive ovaries.

**Daxin is expressed early during embryogenesis and interacts with Arm and Zw3**

In order to study the Daxin protein in embryos we raised antibodies. Endogenous Daxin protein was only detected with the Daxin antibodies by immunoprecipitation followed by immunoblotting (IP-western). Two antibodies directed to distinct regions of Daxin recognize the same set of bands with apparent molecular weight (MW) of 95 kDa (Fig. 5A, predicted MW=81.7 kDa) while their corresponding preimmune sera do not, strongly suggesting that these antibodies detect the Daxin protein. Furthermore, overexpressed Daxin migrates at the identical position (Fig. 5C). At present, the difference in Daxin giving rise to the two bands is not known. It is possible that various phosphorylation states generate the two species of Daxin protein, as is the case for mouse Axin (Willert et al., 1999). Alternatively, the two separately migrating Daxin species could be the A1 and A2 forms of Daxin (described in Fig. 1).

Immunoprecipitation of either Arm or Zw3 co-precipitated the endogenous Daxin protein from embryo lysates (Fig. 5A). Immunoprecipitation of Daxin also precipitated Zw3 protein (Fig. 5B); however, we were unable to detect Arm in Daxin immunoprecipitates from embryo lysates (data not shown).

To confirm that Arm co-immunoprecipitates with Daxin, we overexpressed Daxin under the control of the heat-shock promoter in Schneider 2 (S2) cells. Upon induction of the Daxin transgene, significantly more Zw3 and Arm co-immunoprecipitate with Daxin (Fig. 5C), thus demonstrating that Daxin forms a complex or complexes with Zw3 and Arm.

Finally, we determined the time course of endogenous Daxin expression in the fly embryo. Immunofluorescence staining of whole embryos with the Daxin antibodies produced weak staining (data not shown). Thus, the Daxin protein may be present at very low to undetectable levels or the Daxin specific antibodies do not react efficiently in fluorescence assays. By IP-western, Daxin protein was detectable at low levels during the earliest timepoints (0- to 1-hour old embryos, Fig. 5D), suggesting that some Daxin protein is maternally contributed. At later timepoints, Daxin protein accumulates to higher levels until it reaches a plateau at about 3-4 hours after egg laying (AEL).

**DISCUSSION**

In this paper, we describe the identification and characterization of a Drosophila homolog of mouse Axin,
named Daxin. Daxin negatively regulates Wg signal transduction as revealed by double stranded RNA interference (RNAi) directed disruption of Daxin function and by overexpression of Daxin in the embryo and the larva. Although sequence homology is mainly restricted to the highly conserved RGS and DIX domains, Daxin is a functional homolog of Axin as it is able to bind both Arm and Zw3. Previous genetic screens to identify components of Wg signaling have not yielded mutations in Daxin, which reflects the fact that Daxin is maternally contributed in the embryo (Hamada et al., 1999). This is also suggested by the presence of Daxin protein in early embryos (Fig. 5D).

We took several approaches to study Daxin function. First, we carried out RNAi which yielded the same phenotype (a naked cuticle) as a P-element-induced mutation in Daxin (Hamada et al., 1999). We also attempted to disrupt Daxin function by constructing a dominant negative protein. In Xenopus, it had been shown that deletion of the highly conserved RGS domain produced a dominant negative protein (Itoh et al., 1998; Zeng et al., 1997). Surprisingly, the analogous deletion in Daxin did not interfere with endogenous Daxin function and behaved in an identical manner as overexpressed wild-type Daxin, i.e. production of wg-like cuticle in the embryo and notches along the wing margin. Thus, the RGS domain which interacts with the APC protein (Behrens et al., 1998; Hamada et al., 1999) is dispensable for overexpressed Daxin’s ability to block Wnt signaling.

The discrepancy between the Xenopus and fly data with respect to the RGS deleted Axin may be explained in a number of ways. In analogy to APC, Axin overexpression in various organisms may produce distinct phenotypes. APC overexpression in mouse cell culture is known to down regulate Wnt signaling (reviewed by (Polakis, 1997), while overexpression of APC in Xenopus embryos leads to axis duplication (Vleminckx et al., 1997), a hallmark phenotype of genes that activate Wnt signaling. As another exception to the model of APC being a negative factor, the C. elegans APC homolog Apr-1 has been shown to positively regulate Wnt signaling (Rocheleau et al., 1997). The reasons for the inconsistent phenotypes for APC loss- and gain-of-function are not yet understood.

In support of our findings, Hart et al. have shown that overexpression of human Axin lacking the APC binding domain (i.e. the RGS domain) promotes Axin’s ability to downregulate β-catenin levels (Hart et al., 1998). Finally, although both mutant forms of mouse Axin (Zeng et al., 1997) and Daxin primarily delete the RGS domain, there are subtle

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**Fig. 5.** Daxin protein analysis in embryos and cell culture. (A) Immunoprecipitation of Daxin and co-immunoprecipitation of Daxin with Arm and Zw3. Daxin, Zw3, and Arm proteins were immunoprecipitated from embryo lysates with the indicated antisera or their corresponding pre-immune sera and subsequently immunoblotted with either Rabbit or Rat α-Daxin antibody. Two exposures of the same blot are shown, 1x and 10x, to visualize the Daxin protein in the Arm immunoprecipitate. (B) Zw3 co-immunoprecipitates with Daxin. Daxin was immunoprecipitated from embryo lysates using either rabbit or rat α-Daxin or their corresponding pre-immune sera. The Zw3 band migrates directly above the immunoglobulin heavy chain. (C) Zw3 and Arm co-immunoprecipitate with Daxin in lysates of Schneider 2 (S2) cells. Lysates from uninduced (−) or heat-shocked (+) S2 cells carrying a Daxin transgene under the control of the heatshock promoter were either immunoblotted for Daxin or immunoprecipitated with Daxin antibody followed by Zw3 or Arm immunoblotting. More Zw3 and Arm protein immunoprecipitate with Daxin when Daxin is overexpressed. (D) Timecourse of Daxin expression in embryos. Lysates were prepared from embryos of the indicated age, and Daxin protein was detected by IP-western analysis. The 0- to 1-hour old embryos contain detectable levels of Daxin protein, suggesting that Daxin is maternally contributed. AEL, after egg laying; HS, heat-shock; IgG, immunoglobulin heavy chain; IP, immunoprecipitation; kDa, kilo daltons; Pre, pre-immune serum.
differences in the deletions: DaxinΔRGS deletes the entire RGS domain (amino acids 50-172 in Daxin corresponding to amino acids 89-216 in mouse Axin) while mouse AxinΔRGS deletes part of the RGS domain and some N-terminally flanking sequence (amino acids 124-227 in Axin corresponding to amino acids 87-183 in Daxin). In sum, it remains to be established whether the interaction between Axin and APC is critical for Axin to inhibit Wnt signaling. A genetic analysis of the Daxin gene and identification of mutations that disrupt this interaction will likely provide insights into this question.

As presented here and by Hamada et al., loss- and gain-of-function produce phenotypes consistent with Daxin interfering with Wg signaling. We have extended this analysis to demonstrate that Daxin misexpression can modify a dominant Wg effect in the eye, thus establishing a genetic link between Daxin and Wg. Furthermore, we established that Daxin interferes with an additional Wnt signaling event, namely that of DWnt-2. DWnt-2 triggers pigment cell formation with high efficiency, and this effect can be mimicked by dsh and arm overexpression (C.Y.L., unpublished observations). In addition, Wg can also trigger pigment cell formation but it does so with much lower efficiency than DWnt-2 (C.Y.L., unpublished observations). Thus, Wg and DWnt-2 most likely trigger identical intracellular signaling cascades and Daxin can interfere with these two signals in a similar fashion.

We show furthermore that the Daxin protein, like its mammalian homologs Axin and Conductin, interacts with Arm and Zw3, the fly homologs of β-catenin and GSK3β. Hamada et al. (1999) also demonstrated that Daxin interacts with D-APC, but failed to demonstrate that Daxin can bind Zw3. One possible explanation for this discrepancy is that in vitro translated Daxin is not modified properly to promote an interaction with Zw3. Consistent with this possibility, we have observed that only the slowest migrating Daxin band co-immunoprecipitates with Zw3 (Fig. 5A), suggesting that post-translational modifications, such as phosphorylation, may modulate the interaction between Daxin and Zw3.

Using the RNAi method, we obtained phenotypes that indicate that loss of Daxin function activates Wg signaling. While this result is not unexpected, it should be noted that clear loss-of-function Axin phenotypes have not been described in other organisms, save for the unexplained dominant negative experiments in Xenopus. Our results underscore the potential general use of RNAi methods to obtain loss-of-function phenotypes of Drosophila genes of interest in the fly embryo. These genes may include those that are maternally contributed to the embryo, such as fz (Kennerdell and Carthew, 1998) and Daxin.

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